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Title page

Current Technical Approaches to Brain Energy Metabolism

Barros LF[1], Bolaños JP[2], Bonvento G[3], Bouzier-Sore AK[4], Brown A[5], Hirrlinger J[6], Kasparov S[7], Kirchhoff F[8], Murphy AN[9], Pellerin L[10], Robinson MB[11], Weber B[12]

[1]Centro de Estudios Científicos (CECs), 5110466 Valdivia, Chile

[2]Universidad de Salamanca, CIBERFES, Instituto de Biologia Funcional y Genomica-CSIC, 37007 Salamanca, Spain.

[3]Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA), Département de la Recherche Fondamentale (DRF), Institut de Biologie François Jacob, Molecular Imaging Research Center (MIRCen), CNRS UMR 9199, Université Paris-Sud, Université Paris-Saclay, Fontenay-aux-Roses, France.

[4]Centre de Résonance Magnétique des Systèmes Biologiques UMR 5536, CNRS-Université Bordeaux 146 rue Léo-Saignat, Bordeaux, France.

[5]School of Life Sciences, University of NottinghamNottingham, UK.

[6]Carl Ludwig Institute of Physiology, University of Leipzig, Liebigstr. 27, D-04103 Leipzig, Germany, & Department of Neurogenetics, Max-Planck-Institute for Experimental Medicine, Hermann-Rein-Str. 3, D-37075 Göttingen, Germany.

[7]School of Physiology, Pharmacology and Neuroscience, University of Bristol, University Walk, BS8 1TD, United Kingdom and Baltic Federal University, Kalinigrad, Russian Federation.

[8]Molecular Physiology, Center for Integrative Physiology and Molecular Medicine, University of Saarland, Building 48, 66421 Homburg, Germany.

[9]Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093, USA.

[10]Département de Physiologie, 7 rue du Bugnon, CH1005 Lausanne, Switzerland.

[11]Children's Hospital of Philadelphia Research Institute, Department of Pediatrics, and Department of Systems Pharmacology and Translational Therapeutics, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA.

[12]Institute of Pharmacology and Toxicology, University of Zurich, Switzerland, & Neuroscience Center Zurich, Switzerland.

Running title: pros and cons of experimental methodologies

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Please address all correspondence to:

L. Felipe Barros. Centro de Estudios Científicos (CECs), 5110466 Valdivia, Chile.

Tel: +56 63 2234513

fbarros@cecs.cl

Main points

- Brain energy metabolism in organized hierarchically

- Different organizational levels require different technical approaches

- Technical strengths and weaknesses are made explicit

Keywords

Organization level; spatio-temporal resolution; in vitro; in vivo;

Abstract

Neuroscience is a technology-driven discipline and brain energy metabolism is no exception. Once satisfied with mapping metabolic pathways at organ level, we are now looking to learn what it is exactly that metabolic enzymes and transporters do and when, where do they reside, how are they regulated, and how do they relate to the specific functions of neurons, glial cells, and their subcellular domains and organelles, in different areas of the brain. Moreover, we aim to quantify the fluxes of metabolites within and between cells. Energy metabolism is not just a necessity for proper cell function and viability but plays specific roles in higher brain functions such as memory processing and behavior, whose mechanisms need to be understood at all hierarchical levels, from isolated proteins to whole subjects, in both health and disease. To this aim the field takes advantage of diverse disciplines including anatomy, histology, physiology, biochemistry, bioenergetics, cellular biology, molecular biology, developmental biology, neurology and mathematical modeling. This article presents a well-referenced synopsis of the technical side of brain energy metabolism research. Detail and jargon are avoided whenever possible and emphasis is given to comparative strengths, limitations and weaknesses, information that is often not available in regular papers.

"Each portion of matter may be conceived as like a garden full of plants and like a pond full of fishes. But each branch of every plant, each member of every animal, each drop of its liquid parts is also some such garden or pond."

Gottfried W. Leibniz, Monadology 66-69, 1714

Introduction

Picture a student tantalized by one of our favorite questions: *How is energy production coupled to energy demand? What are the roles of neurons and glial cells in this coupling?* Information processing controls energy metabolism, but *can it also work the other way round? Is epilepsy a metabolic disease? Is energy handling at the core of neurodegeneration?* In search for guidance, she participates in the biennial International Conference on Brain Energy Metabolism (http://cecs.cl/icbem/). After listening to talks and reading posters she realizes that many different techniques are being applied to many experimental models. Each expert uses a different jargon: reversal potential, state 3 respiration, transacceleration, cataplerosis, glutamate enrichment, hyperpolarized, pixel shift, BOLD, default network, etc. One speaker shows single ion-channel recordings. The next one pyruvate carboxylation by mitochondria, and yet another links autofluorescence of mouse hippocampal slices to a genetic disease in humans. She leaves the meeting fascinated and bewildered. *Would the channel behave similarly in situ? Is mitochondrial metabolism sensitive to cytoplasmic context? How does invertebrate metabolism relate to human metabolism?*

Our purpose here is to offer some technological guidance to our prospective colleagues (and to ourselves). Length restrictions preclude covering the ever-expanding technical range, so we apologize for possible omissions. Techniques are described and their strengths are stated briefly. Special attention is allocated to limitations, problems, and ever elusive underlying assumptions. We are not focusing here on the merits of experimental models, which are discussed in the article that introduces this special issue. Before going to the specifics, a theoretical framework may help to explain why such diverse approaches are required and why none of them may claim preference over the others.

Energy metabolism is organized hierarchically

Energy enters the brain as glucose, and leaves it minutes later in the degraded forms of water, CO_2 and heat. The incessant cascade of electrons is somehow harnessed to maintain the complex

structure of the brain and to process information. The science of Brain Energy Metabolism aims to figure out how this is done. It is a relatively young branch of science, stemming from well-established crafts that bring their own concepts, experimental models and techniques, assumptions and flaws: anatomy, histology, physiology, biochemistry, bioenergetics, cellular biology, molecular biology, development, neurology, mathematical modeling, etc. This pleiotropy is necessary because energy metabolism spans multiple organizational levels, from nanometer-scale interactions between intermediate metabolites and enzymes to organ-wide modulation of metabolism by hormones and behavior (Fig. 1). The existence of structural levels and discernible modules within levels is understood by thermodynamics as the expected result of binding forces becoming weaker as small structures accrete into larger ones but also has an evolutionary explanation, as building in modular fashion dramatically reduces error (Simon, 1962). The hierarchical organization of living structures such as the brain makes them amenable to dissection into smaller parts that remain functional. Much fundamental knowledge has been acquired from the study of isolated brain mitochondria, but *which features are context-independent and which are not? To what extent does behavior in culture or in slices represent that observed in vivo?* A given transporter was described and characterized in muscle cells, *does it work the same way in astrocytes? To what extent is it safe to extrapolate from mouse to human?* There are no *a priori* answers to these key questions but a rule of thumb may be applied: the shorter the range of the phenomenon, the more likely it is to be unaffected by context. For example the 18 atoms of glutamate are joined by covalent bonds that withstand 198 ^{o}C. Because covalent bonds are much stronger than the forces that keep organelles, cells and tissues together, we know for sure that the glutamate powder on the shelf is structurally and functionally *identical* to the glutamate released in your visual cortex as you read these words. Thus, applying shelf glutamate to cells in culture is meaningful. Likewise, the ligand specificity of enzymes, transporters and channels is a robust property that may be characterized in a reduced context, i.e. *in vitro*. However, their regulation is most likely not.

Pick your level, pick your technique

The ideal technique would cover the whole organizational range, offering precise control of experimental variables while providing physiologically relevant data that may be reproduced by other laboratories. It should also be affordable and accessible. Of course, there is hardly such technique and trade-offs become necessary. The illustration in Figure 2 shows how some popular approaches distribute over the organizational space. The structure of purified enzymes and transporters is studied by means of X-ray diffraction, while understanding their function may demand complex systems like vesicles. Oxidative phosphorylation is studied by respirometry of isolated mitochondria, where substrates and cofactors are manipulated at will and fast phenomena are followed in real-time, or by respirometry of cell populations, where experimental control and temporal resolution are sacrificed for the benefit of subcellular context and physiological relevance. Cellular and organ level experiments could involve imaging of metabolites concentrations in brain cells *in vitro* and *in vivo* or optical control of signaling in cells of known

phenotype. At the end of the spectrum are minimally invasive techniques like magnetic resonance spectroscopy (MRS), positron-emission tomography (PET) scanning and functional magnetic resonance imaging (fMRI), the only methods that may be used in human subjects.

A The stage: expression, location and interactions of enzymes and transporters

Energy metabolism is distributed across the diverse cellular and subcellular compartments of brain tissue. Division of labor among compartments is evident from clear segregation of key enzyme activities, for example the almost exclusive astrocytic expression of glutamine synthase and pyruvate carboxylase (Martinez-Hernandez et al., 1977; Schousboe et al., 1977; Yu et al., 1983), which inform on the preferential role of these cells in glutamate recycling and anaplerosis. Differences between compartments have also emerged from the study of RNA transcripts in identified cell types acutely isolated from brain tissue by FACS (Cahoy et al., 2008; Zhang et al., 2014). Recently, the possible introduction of expression artifacts by the sorting procedure was minimized by the ingenious expedient of co-culturing neurons and glial cells from different species followed by RNA sequencing and in-silico separation of the transcripts (Hasel et al., 2017).

A.1 How to approach structure based on microscopy

Microscopy has been the technique of choice for investigating the subcellular localization of the various enzymes, transporters and other proteins involved in brain metabolism. In order to make these targets visible under the microscope, different labeling techniques have been used. One group is based on the conversion by the target protein of a chemical substance that becomes a dye accumulating locally within the cell and that can be visualized by bright field microscopy (e.g. DAB staining of cytochrome oxidase(Hevner and Wong-Riley, 1989; Wong-Riley, 1989). The other major group makes use of antibodies that can specifically recognize the protein of interest (Martinez-Hernandez et al., 1977; Vannucci et al., 1997; Choeiri et al., 2002; Lehre et al., 1995; Chaudhry et al., 1995; Pierre et al., 2002; Bergersen et al., 2005; Pierre et al., 2009). After applying a primary antibody specific for the target protein, a secondary antibody directed against the primary antibody and carrying a tag is used to visualize its localization. The nature of the tag can vary and will determine the type of microscopy that will be subsequently used to reveal the presence of the target protein. The tag can be an enzyme which, in the presence of the appropriate substrate, will convert it into a dye that accumulates locally and can be visualized with bright field microscopy (e.g. peroxidase/DAB). If the tag is fluorescent (e.g. FITC or Cy3), then either widefield epifluorescence microscopy may be used, which is more suitable for analysis of tissue cultures and larger overviews, or single- and two-photon laser scanning microscopy, which can provide a 3D diffraction-limited view of tissues. If the tag is a heavy metal atom (silver or gold), then we can use electron microscopy, which increases the spatial resolution power compared to

conventional brightfield or fluorescence microscopy. The fluorescent peptide or protein can then be visualized by fluorescence microscopy (Fig. 3A). Most reagents and antibodies are commercially available or can be obtained from the researcher who produced them. Two limitation of antibody detection, particularly for comparison purposes, are variable affinity and epitope-masking. As an alternative, the mRNA expression of a gene in a given cell type may be evaluated with genetic tools. The idea is to modify the gene of interest and place the sequence coding for a bright fluorescent protein of a particular color (e.g. eGFP, Venus, tdTomato) as a reporter to be expressed under the control of the promoter of the target protein. One example of this type of approach is the expression of tdTomato under the control of the promoter of the lactate transporter MCT1 gene introduced as bacterial artificial chromosome, which revealed preferential MCT1 expression in oligodendrocytes, a finding with consequences for glial-to-neuron lactate shuttling and the pathophysiology of white matter neurodegeneration (Lee et al., 2012).

The main strengths of these various techniques and approaches are their capacity to distinguish the specific cell type within which a particular protein is found and then in which cellular compartment or subcellular domain the protein is localized. From this information, which may be obtained under normal, untreated conditions but also after stimulation or treatment, it is possible to glen information about the participation of a protein in a particular process. In the case of immunofluorescence combined with confocal microscopy, it is possible to determine whether two proteins colocalize, i.e. if they are located close to each other in the same subcellular compartment. The main limitations of these approaches are the specificity and availability of the visualizing reagents. It is essential to demonstrate the specificity of each antibody and sometimes good antibodies are unavailable. Resolution can also be an issue, depending of the degree of localization that is required. For the genetic techniques, discrepancies have been observed between the expression of the native protein and the reporter. Care should be taken to avoid over-expression of reporter proteins as this could cause abnormal expression patterns or toxic effects. Although ideal for structural/localization purposes, these methods are rather limited for the study of dynamic processes.

A.2 Protein-protein interactions, supramolecular complexes, and structural microdomains

Fick's law of diffusion states that the time required for a molecule to diffuse a specific distance increases as a function of distance squared. So, doubling the distance quadruples the time. Thus, protein-protein interactions can reduce the distance between active sites of enzymes and increase the rate of flux of substrates through multistep reactions. In fact, there is good evidence that formation of supramolecular complexes within mitochondria increases the rate of oxidative phosphorylation (Boekema and Braun, 2007) and that proton antennae in neighboring proteins increase the speed of the astrocytic lactate transporter (Stridh et al., 2012). Formation of complexes also likely improves the specificity of some reactions within nanodomains of a cell. For example, interactions with a kinase or ubiquitin ligase might limit the number of proteins phosphorylated/ubiquitinylated upon activation.

Astrocytes have fine processes that contact ~100,000 synapses (Bushong et al., 2002; see Fig. 3A) and endfeet processes that ensheath arterioles and capillaries (Iadecola and Nedergaard, 2007). Thus much like neurons, astrocytes are likely polarized cells in which proteins are selectively sorted into these functionally distinct domains and assembled into multiprotein complexes. A few studies have identified proteins that interact with the Na^+/K^+ ATPase or with glutamate transporters (Genda et al., 2011; Rose et al., 2009; Bauer et al., 2012). Since these proteins are enriched near synapses, this information may help us understand the functional properties of these nano-domains. Similarly aquaporin-4 is enriched in endfeet and forms complexes with other proteins (Nagelhus and Ottersen, 2013). Essentially nothing is known about the mechanisms involved in this sorting and there is still a lot to learn about the complexes that are formed in these nano-domains and their functional significance.

Many interactions are identified by a hypothesis-driven approach: two proteins are found to functionally interact and one tests for protein interactions (for examples, see Robinson and Jackson, 2016). In many cases, antibodies are used to immunoprecipitate a protein of interest and Western blots are used to determine whether an interacting protein is found in the immunoprecipitate (Isono and Schwechheimer, 2010; Kaboord and Perr, 2008). With discovery-based strategies, novel interacting proteins can be identified, information which is used to generate and test hypotheses. Advancements in mass spectroscopy have made it relatively easy to identify proteins in immunoprecipitates. It is also possible to fuse a domain from a protein of interest to glutathione S-transferase (GST) with cloning strategies. The subsequent cDNA is then used to over-express the protein in bacteria, which is then purified and incubated with solubilized tissues of interest. Complexes are then isolated using glutathione-based affinity columns (Harper and Speicher, 2011). Variants of this strategy are used to test whether interactions between two proteins are direct and to map domains of proteins required for a given interaction. Finally one can use yeast two-hybrid based strategies to identify novel interacting proteins. Here, a domain from a protein of interest is fused to part of a transcription factor. Then, the hybrid is used to screen a library of target sequences fused to the rest of the transcription factor and interactions are identified as transcriptional activity (Ito et al., 2001). Candidates can be tested for co-localization in tissues or cells of interest, for example by looking for covariance (Li et al., 2004). If one or both of the proteins are widely distributed, it is possible to determine whether the observed overlap occurs more frequently than that which would occur by chance, by using Monte-Carlo simulation, e.g. (Genda et al., 2011).

There are several issues with any of these approaches. First, it is at least theoretically possible that some interactions form after solubilization. This post-solubilization aggregation is likely due to either electrostatic or hydrophobic interactions and will increase linearly with protein abundance. Sequential rinses with buffers containing different concentrations of salt can reduce these physiologically irrelevant interactions. Second, antibodies used to immunoprecipitate a protein may directly bind to and precipitate non-specific targets. This can be partially addressed by performing ‘reverse immunoprecipitations’ with an antibody against the putative interacting protein. Third, the GST and yeast 2-hybrid approaches require guessing where potential

interacting proteins might bind. Fourth, there are several different types of detergents, including non-ionic, bile acid salts, ionic, etc. One needs to empirically identify the detergents that best solubilize the protein of interest and reduce non-specific binding of proteins in the lysate (for reviews, see Le Maire et al., 2000; Phizicky and Fields, 1995; Garavito and Ferguson-Miller, 2001; Seddon et al., 2004).

It is ultimately important to rigorously demonstrate the relevance of an interaction *in vivo*. Super-resolution microscopy has been improved to the point that the resolving power is in the 10 to 50 nm range. Although this resolution is not sufficient to demonstrate direct protein interactions, it can support the notion that two proteins exist in the same nanodomain. Mapping domains required for an interaction allow for generation of constructs or peptides that will function as dominant-negative inhibitors of the interaction. Of course, if multiple 'interacting' proteins are identified, we must guess which proteins form direct interactions; this is a daunting task because there may be many proteins in a supramolecular complex. For example, the post-synaptic density has about 2,000 proteins (Focking et al., 2016). It is also possible to test for interactions by labeling two putatively interacting proteins with different fluorescent tags. One of the fluorophores is excited and energy is transferred to a second fluorophore by resonance. This technique is called intermolecular Förster resonance energy transfer (FRET) and only detects proteins that are within 10 nm of each other (Sekar and Periasamy, 2003; Jares-Erijman and Jovin, 2003; Pietraszewska-Bogiel and Gadella, 2011).

B The players: function of enzymes and transporters.

B.1 Enzyme activity

Once the location and abundance of an enzyme have been established, the question of function is next to arise. To assess the specific activity of an enzyme time-dependent changes in the concentration of the substrate (or product) need to be determined, during the steady state, in tissue/cell homogenates under specified conditions (Lorsch, 2015), and at a wide range of substrate/ligand concentrations to obtain the main kinetic parameters: half-saturation or Michaelis constant (K_m), half-inhibition constant (K_i), maximal velocity constant (V_{max}) and turnover number (kcat; Purich, 1979; Purich, 1980; Purich, 1982; Brooks et al., 2004). This is essential when predicting metabolic fluxes controlled by enzymes according to substrate concentrations (Kaplan and Colowick, 1955; Herrero-Mendez et al., 2009), tissue damage (Adan et al., 2016) or drug discovery (Widder and Green, 1985; Zhang, 2017).

The specific activity of enzymes is a very useful measurement, and is necessary for understanding the functional fraction. This is important for enzymes that, for instance, are highly susceptible to oxidation of key cysteine residues leading to inactivation, such as mitochondrial respiratory chain complexes (Bolanos et al., 1994; Bolanos et al., 1995). Subcellular fractionation followed by

enzymatic analysis represents a good strategy for estimating specific activity within the natural environment (Murphy and Chan, 2014). The development of techniques for the use of in-gel activity assays for proteins in their native tertiary/quaternary structures, such as blue-native gel electrophoresis followed by NADH dehydrogenase activity for mitochondrial complex I (Murphy and Chan, 2014; Lopez-Fabuel et al., 2016) may also represent progress towards the characterization of enzyme activities in their natural conformation.

The main drawbacks of enzymatic analyses are technical and of interpretation. Technically, the unfeasibility of detecting the substrate or the product forces that couple enzymatic reactions (Purich, 1982), although the product of which - usually NAD(P)H- can be easily determined. Whereas coupling often represents the only available approach for assessing the specific activity of an enzyme, it often generates parasitic reactions (Auld and Inglese, 2004) that mask, usually by overestimation, the actual enzyme activity. When the limitation is due to the low sensitivity of the method to detect the substrate/product, alternative approaches can be implemented to enhance sensitivity. Often, the use of fluorescent, luminescent and radiometric detection methods can overcome the sensitivity problems (Purich, 1980; Simeonov and Davis, 2004; Dahlin et al., 2004), although they require the use of more complex equipment and expensive probes. Kinetic parameters of enzymes responsible for post-translational changes in proteins are difficult to assess as they require more tedious analyses such as Western blotting followed by semi-quantitative estimation of the modified substrate (e.g., phosphorylation, ubiquitination, acetylation, etc; Glickman, 2004), hence complicating the accurate knowledge of the kinetic parameters of the enzyme. Another limitation of this technique is the absence of the physiological environment of the enzyme when its activity is determined. Kinetic parameters of enzymes are determined by adding cofactors and substrates to the reaction mixture at concentrations that might be far from the physiological concentrations in the cell, especially if saturating concentrations are used for determining V_{max}. Furthermore, the specific cellular or subcellular localization of enzymes is not reflected in the reaction mixture, although efforts should be made to mimic the in-cell conditions (Dahlin et al., 2004). For enzymes that are naturally membrane-bound, the use of detergents may help solubilize the protein for easier access of substrates and cofactors, but very often the detergent interferes with light, fluorescent or luminescent detection methods (Simeonov and Davis, 2004). When the detergent cannot be used, due to interferences, the insolubilized membranes cause light scattering, which may preclude spectrophotometric analyses (Brooks et al., 2004). Finally, the specific activity, which represents maximal activity, gives no information about the influence of other enzymes of the pathway on the metabolic flux (Almeida et al., 2004). A well know example is hexokinase, which under physiological conditions work at 10% of its maximal activity, as it is tonically inhibited by its product glucose-6-phosphate (Wilson, 2003).

B.2 Activity of transporters and channels: tracers, fluorescence and electrophysiology

The widespread availability of radioactive tracers at the dawn of the nuclear age led to detailed kinetic understanding of several membrane transporters, well before their molecular

identification. For practical reasons, the best characterized transporters were (and still are) those expressed in erythrocytes, in particular the facilitative hexose transporter GLUT1 (LeFevre, 1948; Widdas, 1954), later found to dominate the glucose permeability of endothelial cells, astrocytes and oligodendrocytes. Radioactive indicator-dilution assays were developed for *in vivo* studies in animals that allowed the kinetic characterization of the transport mechanisms at the blood-brain barrier (Crone, 1965; Yudilevich and Sepulveda, 1976). In human, brain glucose transport parameters could be estimated by combining steady-state measurements of parenchymal glucose concentration obtained by MRS, microdialysis and microelectrodes, with mathematical modeling (Gruetter et al., 1998; Barros et al., 2007). Decades of radiotracer experiments in cultured cells and heterologous expression systems have shown that the selectivity and kinetic properties of transporters for energy metabolites such as glucose, lactate, pyruvate, glutamate and glutamine are insensitive to cellular context. What is less well understood is their regulation, that is, if and how the intrinsic activity and subcellular localization of the transporters responds to the large changes in metabolic demand that accompany neural activity. Excellent in terms of signal-to-noise ratio, radiotracer methods offer limited spatial and temporal resolution, which precludes the study of single cells, complex tissues or rapid regulatory phenomena. In the case of H^+-coupled monocarboxylate transporters (MCTs), the carriers of lactate, pyruvate and ketone bodies, indirect single-cell characterization has been possible by means of intracellular pH electrodes or pH-sensitive dyes like 2',7'-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein (BCECF; Single cell glucose transport determination is possible with the fluorescent glucose analogs 6- and 2-([N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; (6-NBDG and 2-NBDG; Speizer et al., 1985; Yoshioka et al., 1996). They are twice as big as glucose, and are therefore taken by GLUTs at a lower rate (Barros et al., 2009a), a property that can be taken advantage of to achieve real-time measurement of transport activity and thus detect fast regulatory changes (Loaiza et al., 2003; Porras et al., 2004; Porras et al., 2008). Detected by confocal microscopy in brain tissue slices or by two-photon microscopy *in vivo*, they have served to estimate the contribution of neurons and astrocytes to the uptake of sugar (Barros et al., 2009b; Jakoby et al., 2014; Chuquet et al., 2010). Presently, their performance has been surpassed by that of genetically-encoded fluorescent sensors (Section C.3), which provide a stronger signal and a direct readout of the metabolite of interest, avoiding the need for assumptions and controls about analog handling by transporters and enzymes.

A substantial fraction of the brain´s ATP turnover is associated with the recovery of ion gradients dissipated by ion channels during synaptic activity (Harris et al., 2012). In addition, many metabolites possess a net charge and recent studies in glial cells have indicated that some of them may be transported by ion channels (Sotelo-Hitschfeld et al., 2015; Karagiannis et al., 2016). This makes electrophysiology an important tool not just for the manipulation but also for the understanding of brain energy metabolism. The electrical properties of brain cells are typically studied in acute tissue preparations and in cell cultures. In contrast to neurons, which are characterized by action potentials, glial cells have no swift electrical events taking place across their membranes. In addition, the plasma membranes of astrocytes and oligodendrocytes are extremely leaky due to high background activity of potassium channels. There are also no fast

excitatory or inhibitory synapses on astrocytes or oligodendrocytes and therefore stimulation of an afferent pathway commonly used for activation of neurons cannot be used to synchronously "fire" them. For these reasons electrical recordings are less commonly used in studies of these cells. Recordings with extracellular electrodes can detect stimulus-evoked compound action potentials, CAPs (Stys et al., 1991), where changes in the extracellular ion distribution due to glial transporters do play a role, but it is very hard to separate the glial component from the contributions of other tissue elements. From the metabolic point of view, the *in vitro* recording of CAPs offers the advantage that the tissue can be maintained for extended periods in a perfusion chamber with temperature, degree of oxygenation and nutrient supply controlled by the experimenter. In a homogeneous tissue such as the optic nerve, the CAP represents the response of a common neural cell type, central white matter myelinated axons. However in less homogeneous tissue e.g. the hippocampus, the recorded response is from heterogeneous cell types is recorded, thus the response may not be a true reflection of the contribution of individual neural cell types (Cater et al., 2001; Brown et al., 2003). *In vitro* recordings of acutely isolated tissue may be problematic with regards to the diffusion barrier created by large pieces of tissue, where inadequate diffusion of oxygen and glucose may lead to anoxic and/or hypoglycemic conditions at the center of the tissue (Tekkok et al., 2005).

Recordings with sharp glass electrodes are possible for large glial cells (Deitmer, 1992). Intracellular recordings in patch-clamp mode seem to be more suitable for studies of astrocytes, which are usually much easier to patch than neurons in spite of their small size. Using the patch clamp approach it is possible to perform an accurate pharmacological characterization of receptors expressed by astrocytes (Lalo et al., 2008; Palygin et al., 2011). However, such experiments work best when astrocytes are isolated because it is not always easy to localize the site of action of the drugs when applied to tissues. Experiments *in vitro* allow tight control of the perfusion media including concentration of metabolites and drugs and the recordings are much more stable. However, astrocytes are very sensitive to stress, which is inevitable during preparation of slices and they easily convert to a "reactive" phenotype that may have a somewhat different physiology to the naïve cells *in situ*. In addition, metabolic conditions in a slice never correctly mimic conditions in vivo. Most acute slice experiments are performed in solutions bubbled with 95% O_2/5% CO_2 mix, resulting in hyper-oxygenation of the superficial cells. For some experiments this is a problem, as shown for example in studies of neuro-vascular coupling where the O_2 level was eventually reduced to 20% (Hall et al., 2012). However, using lower oxygen can easily lead to hypoxia in the center of a moderately thick slice (300 µm). In a similar vein, virtually all standard slice work is performed in solutions containing 10 mM glucose or more, while brain tissue glucose is about 1mM (Barros et al., 2017). This overloads superficial astrocytes with glucose. Finally, cultured astrocytes are ideally suited for patch recordings but culturing affects the morphology, physiology and metabolism of these cells, factors that can be ameliorated by co-culturing with neurons (Mamczur et al., 2015; Hasel et al., 2017). In summary, patch clamp is perhaps the best electrophysiological approach for studying astrocytes, but caution is required when extrapolating from data obtained on isolated astrocytes. It should also be remembered that while recording with patch electrodes, we artificially impose on the cell our ideas about the

intracellular concentration of ions and metabolites because pipette solutions rather quickly dialyse into the astrocyte. Thus, our conclusions about the currents in these cells are only as accurate as our knowledge of their native intracellular ion composition.

B.3 Optogenetic actuators: manipulating metabolism

Optical control of electrical and signaling events in brain tissue *in situ* is in principle highly attractive because it seems to be non-invasive, fast and can be targeted to individual cells and cellular populations. Although light-sensitive proteins that affect cellular functions in plants have been known of for a long time, a major shift in neuroscience took place around 2003-2006 when it was demonstrated that such proteins can be expressed in mammalian neurons and used to activate them (Nagel et al., 2003; Boyden et al., 2005; Bi et al., 2006). Channelrhodopsin-2 (ChR2), which was (and still is) the most popular member of this family behaves as a light-gated channel permeable to sodium, potassium, protons and Ca^{2+}. Manipulating sodium conductance presents an obvious way to depolarize neurons and trigger action potentials on demand. This is by far the most common use for this protein, which can be targeted to specific populations of cells using molecular and genetic approaches, and it will be very interesting to exploit it to investigate the role of specific neurons on local energy metabolism. ChR2 has also been used to activate astrocytes (Fig. 3F; Gourine et al., 2010), where it triggered Ca^{2+} elevations, though this required an application of several seconds of pulsing blue light (NB: constant blue light is a notorious source of artifacts). The Ca^{2+} increases were largely dependent on thapsigargin-sensitive internal Ca^{2+} stores (Figueiredo et al., 2014; Perea et al., 2014). ATP release was also documented from ChR2-stimulated astrocytes (Gourine et al., 2010). Many studies in the meantime have used ChR2 to activate astrocytes, though it should be noted that this is not an ideal tool for controlling these cells. A significant Na^{+} influx most likely affects multiple Na^{+}-dependent transporters on astrocytes and the downstream effects become hard to disentangle. Prolonged ChR2-mediated activation leads to acidification of astrocytes due to a build-up of lactate (Tang et al., 2014). Since ChR2 does not mimic any obvious endogenous physiological mechanism operating in astrocytes, it is reasonable to explore alternatives. Attempts to use exotic approaches such as the light sensitive proton pump Arch have also been made (Poskanzer and Yuste, 2016; Beppu et al., 2014), but the actions of this protein on astrocytes are even less clear than those of ChR2. Light-activated G-protein coupled receptors (GPCR) have been used (Tang et al., 2014) with the assumption that via this route events can be evoked that are similar to the native signaling in the brain where astrocytes are mainly controlled via GPCRs. These constructs however need to be used with caution because, when expressed at high level, GPCRs have a tendency to signal without any external trigger. Thus, overexpression, commonly used in many experiments, can be seen as a problem. An interesting new approach is to use a dimerization strategy for optical activation of signaling, for example activities of kinases. For astrocyte research a construct has been introduced that triggers Ca^{2+} entry via the ubiquitous CRAC channels (Kyung et al., 2015). Even though this tool may not be fast enough for triggering really rapid events, it seems that for many cases it can be advantageous, because at least it is a clearly defined mechanism for increasing astrocytic Ca^{2+}.

To summarize, the use of optogenetics for the study of brain energy metabolism is just starting, with a promising future of ever more specific and powerful ways to manipulate individual cells.

C. The play: metabolite concentrations and fluxes

C.1 Respirometry

Neural activity is tightly coupled to local fuel oxidation. The oxygen consumption rate (OCR) is therefore highly valuable information when characterizing cellular bioenergetics or diagnosing diseases. After decades of productive use for research and teaching, the estimation of oxygen consumption with manometry (Warburg, 1928) was superseded by easier and more accurate, real-time determinations by using polarography (Clark et al., 1953). Whilst the traditional Clark-type electrode has delivered most of our current knowledge of mitochondrial bioenergetics, it has disadvantages (Zhang et al., 2012). Oxygen often leaks from the chamber through junctions and holes used for the administration of drugs and inhibitors; assembly of the equipment is tedious, time consuming and requires frequent calibration; the electrode is easily oxidized or damaged, and thus has a short lifespan; the need for continuous stirring to mix the cell or mitochondrial suspension usually harms the sample, in particular when analyzing isolated intact mitochondria; and since several of the inhibitors used to characterize the contribution of mitochondrial complexes to oxygen consumption are lipid soluble, the chamber needs to be thoroughly washed between samples. Whilst the chamber size is often fixed, the internal volume can be adjusted between 0.3-2 ml. However, the best results are often obtained with large sample volumes (usually, 1 ml), thus requiring large amounts of biological material for the analysis. This makes the system unsuitable for screenings. The appearance of multiwell plate respirometers has fired up the field (e.g. Agilent technologies/Seahorse Bioscience, Presens). These devices are built to simultaneously determine OCR and extracellular acidification rate (ECAR) in 24- or 96-well plates of adherent rather than suspended cells. OCR is determined using an oxygen-sensing fluorophore instead of the traditional oxygen-consuming electrode, and ECAR, with a pH sensor. Though popular, the high cost of both the instrument and the single-use plates are a disadvantage. In addition, ECAR is often thought to reflect glycolysis rate because the release of lactate acidifies the extracellular medium. However, caution should be taken because the CO_2 released by the tricarboxylic acid activity significantly contributes to extracellular acidification (Mookerjee et al., 2015; Mookerjee et al., 2017). The Clark electrode and the fluorophore-based multi-well instruments are highly valuable tools for characterizing aerobic metabolism in biological samples. Choosing one or the other depends on sample type and on flexibility in the study design. For instance, the Clark-type electrode chambers are very useful when the sample size is not limiting, as it provides more accuracy and time resolution, but the method is time consuming, such that only a few samples can be assayed in a day. This approach is also suitable for tissue slices or

isolated mitochondria in suspension, as well as intact or permeabilized cells in suspension. It should be noted that harvest of neurons from a culture dish for assay in suspension leaves behind the neuronal processes and the mitochondria within them. With both assay platforms, a large number of substrates, inhibitors or uncouplers can be used to better characterize the bioenergetics of the sample. Selective permeabilization of the plasma membrane allows control of substrate delivery to the mitochondria, and avoids the need for isolation of mitochondria from cultured cells (Divakaruni et al., 2014). The multi-well apparatus, in contrast, is very useful for accurate screenings in intact adherent cells, permeabilized adherent cells, or isolated mitochondria and requires a smaller sample size (around 1-3 x 104 cells/well or 1-3 micrograms of isolated mitochondria/well; Divakaruni et al., 2017; Rogers et al., 2011). This is particularly important when determining OCR in highly differentiated cells, such as neurons, given the low yield in these preparations. Note that multi-well respirometry is routinely employed in the absence of bicarbonate, a substrate of the anaplerotic enzyme pyruvate carboxylase and a regulator of astrocytic glycolysis (Ruminot et al., 2011). Multiwell respirometry is also constrained in terms of temporal resolution, with a typical rates integrated over 2-5 minutes. One limitation common to both oxygen electrodes and multi-well methods is the need for homogenous cell populations, which is not ideal for the study of brain cells, as neurons and astrocytes foster their mutual functional and metabolic differentiation (Barres et al., 1990; Mamczur et al., 2015; Hasel et al., 2017). A limitation of the use of isolated brain mitochondria in either apparatus is the fact that the mitochondria arise from multiple different cells types, thus specific information on the function of neuronal versus astrocytic mitochondria is lost in this approach. However, respirometry can be performed on preparations of synaptoneurosomes, which are resealed synaptic vesicles created upon homogenization of brain tissue that contain mitochondria from synaptic terminals (Choi et al., 2009).

C.2 Extracellular microdialysis and enzyme-based probes

Given that the interstitial fluid is the medium of exchange between blood, the cerebrospinal fluid and the intracellular compartments of neuron and glia (Ransom, 2009), the ability to measure interstitial metabolites is crucial. However, the space left between brain cells is only 20 nm wide, which makes probing its composition a major technical challenge. Dialysis probes have been used to measure metabolic substrates from awake, moving animals (Brown et al., 2011). Placement of the dialysis probe requires complex surgery from which the animal recovers. The probes can be permanently attached to the animals' skull with the sensing component extending into the brain parenchyma. The probe is guided through the brain parenchyma until a desired location is reached, which creates considerable localized damage and forms a 3rd space that fills with the contents of damaged cells, and whose composition clearly does not represent the baseline interstitial fluid. However this perturbation dissipates over time as local repair mechanisms tend to normalize the 3rd space contents. The interstitial fluid is sampled by applying backpressure, such that a small volume is drawn up into the probe (5-10 µl) to be later analyzed by HPLC. Repeated samples can be withdrawn over extended periods of time (days to weeks) thereby facilitating

longitudinal studies. Advantages of this technique include its use for extended periods and the accuracy of HPLC analysis. Some disadvantages are that it does not provide real-time information, it disrupts the tissue, has low spatial resolution. It is a hotly debated area whether the samples reflect the real environment in the interstitial space.

Real-time measurements of brain interstitial fluid can be obtained with enzyme-based microelectrodes. Probes can be as fine as 7 μm in diameter with the active tip between 100 and 500 μm in length. The greater the surface area of the tip the greater the temporal resolution. Such probes are useful for recording from homogeneous brain structures such as isolated optic nerve (Yang et al., 2014b) and hippocampus (Fig. 3E; Newman et al., 2011; Frenguelli et al., 2003). However, as the probe records the aggregate signal from the entire tip area, care should be taken in interpreting data acquired from brain areas with heterogeneous cell populations. Those probes currently available can sense a multitude of compounds of interest to metabolic studies, including ATP, adenosine, acetylcholine, glucose, lactate, D-serine, choline, glutamate. The sensors can be calibrated prior to the experiment and are thus able to provide real-time estimates of concentrations, and they can be used for both *in vivo* and *in vitro* studies. A great advantage of modern commercially available sensors is that several probes may be used simultaneously to measure the concentrations of multiple metabolically relevant compounds e.g. glucose and lactate, ATP and adenosine (Fig. 3E; Frenguelli et al., 2007; Newman et al., 2011). Microsensors can be used in parallel with other real-time techniques, e.g. electrophysiology (Brown et al., 2012; Yang et al., 2014b) and genetically-encoded intracellular nanosensors (Machler et al., 2016). The power of combining functional assays with the real-time measurement of metabolic substrates has proven to be very effective in determining key processes in both grey and white matter.

C.3 Genetically-encoded fluorescent nanosensors for metabolites

Metabolite nanosensors are chimeric proteins that change their optical properties upon binding a ligand (Tsien, 2009; Fehr et al., 2003). Their specificity is conferred by a recognition domain, such as a bacterial periplasmic protein or a transcription factor, and their fluorescence by one or two fluorescent proteins, such as GFP, mTFP or Venus. Many of them are based on intramolecular FRET. Expressed in cellular compartments, the nanosensors are detected by microscopy: wide field, confocal or two-photon. In brain cells, measurements have been reported for glucose, glutamate, ATP, ATP/ADP, NADH/NAD^+, lactate and pyruvate, reviewed in San Martín et al., 2014b. Most sensors are easily available through the non-profit repository Addgene. The main strength of these tools is resolution, which for high-end microscope setups is in the order of seconds and micrometers. Thus, they provide a real-time estimation in single cells. Being genetically-encoded, they can be targeted to organelles and subcellular domains (Pendin et al., 2017). Their most common and robust use is to report metabolite fluctuations in before-and-after experiments, for example when characterizing the effect of a physiological or pharmacological perturbation (e.g. see Fig. 3G). Using standard transfection or viral expression systems, sensor concentration is about 10 μM (Miyawaki et al., 1999), which is lower than the physiological concentration of most

metabolites and therefore metabolite sequestration and slow dissociation kinetics are not usual concerns. An important exception is NADH, which is in the nanomolar range.

As they are proteins, nanosensors are potentially susceptible to their physical environment. Some of the early versions were sensitive to pH, which could be corrected by parallel pH measurements. Their main weakness is that they are difficult to calibrate. One important caveat, which is not always explicit, is that it is not safe to draw conclusions from differences in intensity between cells. Even for FRET-based and other ratiometric sensors, the readout is affected to some extent by expression level due to non-linearities in the optical path. To compare cells, a calibration is therefore required. The simplest one-point calibration is achieved by forcing a reading at one end of the binding curve (i.e. zero or saturation) and then using the affinity constant and maximum fluorescence change determined *in vitro* to extract concentrations by interpolation (Barros et al., 2014). If both ends of the saturation curve are accessible, a more robust two-point calibration protocol becomes feasible, where only affinity needs to be assumed as shown for the pyruvate sensor (San Martín et al., 2014a). Avoiding assumptions by recurring to a full *in situ* calibration is possible (Bittner et al., 2010) but is seldom practical, as it demands harsh permeabilization and/or metabolic inhibition procedures that may perturb the sensor itself. When quantitative data are available, nanosensors may be used to measure absolute flux by means of inhibitor-stop protocols (Bittner et al., 2010; San Martín et al., 2013; San Martín et al., 2014a). As both calibration and quick transporter inhibition are needed, this kind of assay is currently restricted to cells in culture and in slices. Inhibitor-stop methods assume that there is no quick adaptation to the inhibition, which has to be evaluated on a case-to-case basis.

Nanosensing is an emerging technology and probes for other metabolites are surely on their way. One example is a sensor for $NADP^+$ (Cameron et al., 2016), which may help in tackling the cryptic pentose phosphate pathway (Bouzier-Sore and Bolanos, 2015). In parallel, existing sensors are being improved and extended in terms of brightness, affinity, dynamic range and spectra (e.g. red-shifted for deeper tissue penetration by reducing scattering). One important objective at hand is the generation of extracellular sensors to approach the brain interstice in non-invasive manner.

C.4 *In vivo* optical imaging: from autofluorescence to genetically-encoded sensors and transgenic animals

Brain energy metabolism has been the focus of studies since optical imaging methods began in the 1960s (Chance et al., 1962), where changes in tissue UV autofluorescence observed upon altering respiration *in vivo* were interpreted as changes in the NAD^+/NADH ratio (Mayevsky and Chance, 1975). This and its relative flavoprotein autofluorescence imaging have been widely applied (Shibuki et al., 2003; Weber et al., 2004; Shuttleworth, 2010; Reinert et al., 2011; Kasischke et al., 2004). In the early reports, the dynamics of hemoglobin oxygenation was discussed, a phenomenon that was later exploited by Grinvald and colleagues to map brain activity (Malonek and Grinvald, 1996), and which paved the way for the development of blood oxygenation level dependent (BOLD) fMRI (Ogawa et al., 1990). Oxy- and deoxyhemoglobin can be discerned

optically due to their differential absorption spectrum. However, the intrinsic optical contrast mechanisms have only marginally helped to generate mechanistic insight into cell-specific metabolic processes, because the signals are difficult to discriminate from each other, and hard to quantify and interpret (see Box 1). After their successful use in cultured cells and brain tissue slices (Section B.4), fluorescently labeled substrates were used to investigate energy metabolism in combination with two-photon microscopy (Denk et al., 1990; Zipfel et al., 2003). Using the non-metabolized fluorescent glucose analog 6-NBDG, an acute increase in glucose uptake was detected in astrocytes but not in neurons of the somatosensory cortex of stimulated rats (Chuquet et al., 2010). However, the optical properties of NBDGs are not favorable because of a very low two-photon cross-section. Alternative fluorescent glucose analogues were developed (Kovar et al., 2009; Tian et al., 2009) and used *in vivo* (Lundgaard et al., 2015), but the data remain difficult to interpret because some are hardly transported due to high molecular weights of 1.3 kDa (Kovar et al., 2009; Lundgaard et al., 2015). The advent of genetically encoded sensors for energy substrates (Section C.3) promises to be a significant advance for the field of brain energy metabolism, as they offer cellular resolution and real-time measurements (Fig. 3G; Lerchundi et al., 2015; Sotelo-Hitschfeld et al., 2012; Machler et al., 2016). In combination with advanced viral expression vectors and hopefully transgenic mice (see below), these sensors are particularly well suited for *in vivo* research using two-photon microscopy. Quantitative measurements of partial pressure of oxygen (pO_2) in vivo can be obtained with phosphorescence lifetime imaging microscopy (PLIM), in which a phosphorescent dye produces a decaying light signal after a short illumination. This decay is shortened by the presence of oxygen and its half-life can be translated to pO_2 in absolute units. New phosphorescence probes were recently developed that can be two-photon excited (Esipova et al., 2016; Esipova and Vinogradov, 2014), which greatly widens the range of *in vivo* applications (Lecoq et al., 2011; Lyons et al., 2016; Parpaleix et al., 2013; Sakadzic et al., 2010; Sakadzic et al., 2011). Until recently, high resolution imaging was only possible in anesthetized animals. A general concern for *in vivo* optical imaging and *in vivo* experimentation are the effects of anesthesia on metabolic processes. For this reason, an increasing number of laboratories are now using head-restrained awake rodents even with two-photon microscopy at single cell resolution.

Transgenic animals for metabolic imaging. The study of brain structure and function has been significantly facilitated by the availability of transgenic reporter animals, from nematodes to mammals, contributing for example, to the discoveries of the neurogenic potential of embryonic glia (Malatesta et al., 2003) and microglial motility (Nimmerjahn et al., 2005). More recently, genetically encoded sensors have been used as biosensors to visualize transmitter- and behavior-evoked Ca^{2+} signals (Akerboom et al., 2012; Paukert et al., 2014). In transgenic animals the sensors are expressed permanently at constant levels over generations, a distinct advantage over viral transduction, which requires injection of each animal, with the possibility of tissue injury and inflammation. In addition, transgenesis provides a higher degree of flexibility regarding cell-specific expression and size of the reporter construct. The generation of transgenic mammals is however time consuming, labor intensive and expensive. Another important drawback is the scarcity of appropriate promoters to reach sufficient expression levels in specific cell types, but strategies have been developed to address these limitations (Hirrlinger et al., 2009; Madisen et al.,

2015). To our knowledge there are only two transgenic mouse models to measure metabolites in brain tissue at this time: one that ubiquitously expresses a nanosensor for pyruvate (Bulusu et al., 2017) and the other specific for neuronal ATP (Fig. 3G; Trevisiol et al., 2017). The pyruvate sensor was used to reveal the spatiotemporal dynamics of tissue explant glycolytic activity during development and the ATP sensor allowed imaging, for the first time, of the frequency-dependent energy demand of optic nerve axons.

The age of metabolism studies using transgenic animals is just beginning. Novel genetic tools such as CRISPR/Cas9 (Cong et al., 2013; Wang et al., 2013) facilitate particularly the generation of mouse lines with loxP-flanked transcriptional "stop" sequences followed by nanosensors targeted to a single well-defined gene locus (e.g. at ROSA26). Combinatorial breeding of mice with expression of inducible and cell-specific Cre-DNA recombinase and ROSA-nanosensor mice will provide promising opportunities to explore the many facets of energy metabolism in the mammalian brain. Transgenic *C. elegans* and *D. melanogaster* lines have been generated that express an ATP sensor (Tsuyama et al., 2013). Recently, fruit fly lines selectively expressing pyruvate and glucose sensors in mushroom body neurons helped to show that energy metabolism can play an instructional role in long-term memory encoding (Plaçais et al., 2017), a role that appears to extend to behavior (Li-Byarlay et al., 2014). Another fruit fly study expressed a glucose sensor to characterize the delivery of glucose to brain cells (Volkenhoff et al., 2017). Short life cycle, relatively inexpensive experimentation and the availability of powerful tools for genetic manipulation, renders worms and flies very attractive for comparative studies of brain energy metabolism using imaging probes.

C.5 2-deoxy-[^{14}C]glucose and [^{18}F]fluorodeoxyglucose methods for the measurement of local glucose utilization

2-Deoxy-D-glucose (2DG) is an analogue of glucose (a hydrogen atom replaces the hydroxyl group on the second carbon) and this single structural difference is responsible for the chemical properties that make 2DG so suitable for the measurement of the rates of glucose utilization in the brain *in vivo*. 2DG is transported in the cell and metabolized qualitatively like glucose but only through the first step of the glycolytic pathway to provide 2DG-6-phosphate (2DG-6P). Since 2DG-6P is not metabolized further to a significant extent, its rate of accumulation is proportional to the amount of glucose used by the cell. Using an appropriate mathematical model of enzyme kinetics and [^{14}C]-labeled 2DG associated with autoradiography (a technique using X-ray films to visualize radioactive molecules with a spatial resolution of approx. 50 µm), the [^{14}C]-2DG method provides quantitative metabolic maps (in µmol of glucose/g/min) of the brain in which regions of altered activity can be easily visualized (Sokoloff et al., 1977). This method can be used in different species, yields massive amounts of data and has provided unequivocal evidence that energy metabolism is linked to local functional activity. This method was adapted for use in humans with [^{18}F]fluorodeoxyglucose (FDG) and a positron emission tomography (PET) scanner (Reivich et al.,

1979), which remains useful as a tool for mechanistic investigation of brain energy metabolism in animals (Zimmer et al., 2017).

Absolute quantification of glucose utilization is possible thanks to the use of ^{14}C-2DG, but many laboratories lack authorization to work with long life radioactive isotopes. The operational equation based on the original model requires plasma glucose (glucose oxidase method) and [^{14}C]-2DG concentrations (liquid scintillation counter) to be sampled frequently during a 45-min period, for which intravenous and intra-arterial catheters are inserted. While this is feasible in large animals, this can represent a technical limitation in mice, the choice animal model in the neurosciences. Also, the method requires that the brain of the animal is processed for autoradiography soon after completion of the experiment. Therefore only one measurement is possible per animal. The brain is cut entirely into 20 µm-thick sections with a cryostat and dried sections mounted on coverslips are left in contact with an X-ray film or phosphor screens. After exposure data can be read out with means of a specific image analysis system or phosphor-imager. The method cannot measure individual cells so that it is not possible to separate, for example, the contribution of neurons and glial cells. 2DG and to a larger extent FDG (high electronegativity of the fluorine-18 atom) do not have the same affinity as glucose for either glucose transporters or hexokinase. For quantitative analyses, these differences must be corrected for by the lumped constant, a parameter present in the operational equation used to determine the local rate of cerebral glucose utilization. While the operational equation has been thoroughly validated for its use in physiological conditions, it may not apply in specific pathophysiological conditions in which cardiovascular function or blood glucose levels are altered. FDG remains the most frequently used tracer in PET imaging, particularly for the detection and staging of tumors, in spite of the low spatial resolution of PET, the relative invasiveness of the method and the radiation dose due to the injection of [^{18}F]-FDG. The use of fMRI-BOLD (see Box 1) now represents the gold standard to study the human brain at rest and during increased functional activity.

Even if the 2DG/FDG method cannot provide information on the fate of glucose downstream of the first hexokinase step, it is as yet the only one that provides quantitative measurement of local glucose consumption in various structures of the brain. The recent development of computerized procedures for the automatic acquisition and 3D reconstruction of postmortem autoradiographic volume images now permits voxel-wise statistical methods to 3D-analyze the differences in glucose use in different groups of animals (without *a priori* identification of Region-of-Interest), as is done in humans (Fig. 3B; Dubois et al., 2010; Boussicault et al., 2014).

C.6 Magnetic Resonance Spectroscopy

Combined with MRI, MRS is a powerful technique that permits simultaneous visualization and quantification of dozens of metabolites contained in the brain area studied. The major advantage of MRS though is the possibility to obtain metabolic information in a non-invasive manner.

Nuclear magnetic resonance arises from the interaction between atomic nuclei that possess an intrinsic angular momentum, or spin, and a magnetic field. This interaction is a very weak one that confers a low sensitivity on this technique, depending on the nucleus considered, the signal being stronger for ^{1}H and much weaker for ^{13}C, for example. Most of the *in vivo* studies are thus ^{1}H-MRS. Metabolites are analyzed in a voxel, or volume of interest, located on the MR image acquired just before MRS (Fig. 3C). Accuracy is therefore strongly dependent on the immobility of the animal, which can be controlled by performing another MRI just after MRS. Anesthetic choice is also very important, especially for metabolic studies, since many drugs have an impact either on the vascular response or on metabolite contents (Horn and Klein, 2010; Tchaoussoff et al., 1991). Note that only metabolites present at a concentration higher than 1mM may be detected by MRS. Moreover, spatial and temporal resolutions are rather low, for voxels are in the mm^3 range, thus preventing discrimination at the cellular level, and, to get enough signal-to-noise ratio, acquisitions are performed over minutes rather than seconds. This temporal resolution is even lower with ^{13}C-MRS, which has a detection level 4 orders of magnitude lower than that of ^{1}H. Therefore, the use of ^{13}C-labeled precursor is a prerequisite to follow brain metabolism. ^{13}C-labeled metabolites are infused in the blood circulation of the animal, at a rather high concentration, which leads to hyperglycemia with the main ^{13}C-labeled precursor used, i.e. ^{13}C-glucose, a point that is often neglected but has an impact when studying brain metabolism. Moreover, this labeled substrate, infused in the blood circulation, is not only metabolized by the brain but also by other organs, such as the liver for example, which may scramble the position of the ^{13}C in some metabolites that go back to the blood and then to the brain, and thus complicates the analyses at the cerebral level. However, ^{13}C-MRS is a powerful technique since the fate of the ^{13}C can be followed and the exact labeled carbon positions of each detected metabolite can be determined and quantified, allowing the pathway taken by the ^{13}C-labeled precursor to be traced back (Rodrigues et al., 2013; Rothman et al., 1999). Even if this technique does not reach cellular resolution, it can distinguish between a predominant neuronal or astrocytic metabolism. This is based on the principle that neurons and astrocytes differentially express some enzymes; e.g. glutamine synthase and pyruvate carboxylase.

Several approaches have been developed to overcome the low sensitivity of MRS. First, since the signal is directly proportional to the field strength, companies develop magnets with increasing magnetic fields. Secondly, the ^{13}C weak signal can be detected by ^{1}H-MRS using proton-observed carbon-edited (POCE) sequences. Finally, improved sensitivity for ^{13}C can be attained with polarization transfer experiments, which partly transfer energy from electron to nucleus, increasing the signal by 10,000 times, but only for some tens of seconds. Even if MRS is not a very sensitive technique, this relative weakness is also its strength, enabling NMR to study living organisms without significantly disturbing them and thus yielding detailed metabolic information non-invasively, which allows longitudinal studies (Stagg and Rothman, 2014; de Graaf, 2013).

In parallel to *in vivo* MRS, *ex vivo* NMR spectroscopy is also often used to explore details of brain metabolism (Fig. 3D). One of the biggest advantages of *ex vivo* NMR spectroscopy is achieving very high-resolution spectra and metabolic profiles. Spectra can be obtained either on perchloric acid

extracts of cell cultures or tissues. Alternatively, with the use of special NMR probes such as HRMAS (high resolution magic angle spinning) it is now possible to achieve the same high spectral resolution on biopsies (without any biochemical treatment) as at obtained in liquid state NMR (extracts). The very recent development of the micro HRMAS probe (HRμMAS) allows for high quality spectra on nanoliter-sized samples (Duong et al., 2016). An important issue when performing *ex vivo* analyses is to completely stop metabolism before acquiring the spectra. For cell cultures, the cellular layer is quickly rinsed with cold PBS (to avoid any contamination with medium metabolites) and cells are further either frozen with liquid nitrogen or directly extracted with perchloric acid. For brain biopsies, the only technique to avoid any post-mortem metabolism is to use focused microwaves. In less than one second, brain temperature reaches 70-80 °C, a temperature at which proteins are denatured and thus all enzymatic activities stop. The brain can be then taken out of the skull and a precise brain region can be either directly analyzed by HRMAS NMR spectroscopy or further processed with perchloric acid extraction of the metabolites for later liquid state NMR analyses.

The technical considerations described above are valid for all observed nuclei (^{1}H, ^{13}C, ^{31}P, etc). For ^{13}C, the use of ^{13}C-labeled precursors is also needed (see *in vivo* spectroscopy paragraph). However, natural abundance can also be observed with *ex vivo* NMR spectroscopic studies, compared to *in vivo* studies, since long time acquisition (several hours) can be performed. Finally, the high spectral resolution of ex vivo NMR spectroscopy permits analysis of what is called homonuclear spin coupling. When several ^{13}C are located on the same molecule, the resonance of the observed carbon is split and multiplets will appear. The analysis of these homonuclear spin-coupling patterns represents a unique advantage over other metabolic monitoring techniques such as radioactivity (Rodrigues et al., 2013).

C.7 Metabolomics, mass spectrometry and stable isotope tracing

Metabolomics. Cataloguing the complement of metabolites in a biological sample can lend important insight, particularly in the search for disease biomarkers. Mass spectrometry and proton NMR are generally the methods of choice for metabolomics (Dumas and Davidovic, 2015). Proton NMR, a faster technique that can have a simpler sample preparation than mass spectrometry, measures metabolites in the low micromolar range, and has been used extensively in the characterization of CNS disorders (see Section C.6 and Oz et al., 2014), whereas mass spectrometry can detect picomolar and even femtomolar quantities of metabolites with time-of-flight and quadrupole time-of-flight detection systems. However, mass spectrometry typically involves a more complex sample extraction, derivatization of the metabolites to facilitate detection, and separation techniques prior to injection of a sample into the mass spectrometer. In general, methanol:chloroform extraction allows separation of polar and non-polar metabolites, and gas chromatography is used to further separate polar compounds, whereas liquid chromatography is used to separate non-polar metabolites. Following separation, the samples are ionized, and are separated and/or further fragmented in the mass spectrometer that detects the

species based upon their mass/charge (m/z) ratio. Each metabolite generates its own pattern of peaks in the m/z spectrum, and the peak heights generally correlate with metabolite concentration. Huge quantities of data are generated by mass spectrometry, thus sophisticated systems biology data analysis tools are used to interpret the spectra, to detect differences between samples, and to define changes in patterns of related metabolites and metabolic pathways. Such approaches have been used to identify changes in the metabolome in a wide variety of neurodegenerative diseases in humans and in animal models (Dumas and Davidovic, 2015).

A particular challenge with metabolomics is to determine the meaning and significance of the multitude of findings with respect to cell and tissue health or to disease etiology. Relatively low throughput and intensive data analysis, as well as the high cost of state-of-the art LC- and GC-mass spectrometers present additional challenges. In metabolomics, spatial and subcompartmental information is lost except for the regional information provided by NMR-based spectroscopy. However, mass spectroscopy imaging is beginning to address cellular, and even subcellular resolution of metabolites using fluorinated nanoparticles in nanostructure-initiator mass spectroscopy and related approaches (Kurczy et al., 2015). If a metabolite concentration increases, it is unclear whether the change arises from enhanced production, or decreased consumption of the intermediate, as metabolomics is generally based on data gathered from a single snapshot-in-time. Thus, whether or not there are alterations in the flux of a metabolite through a metabolic pathway cannot readily be discerned. Computational flux balance can yield estimates of metabolic flux without labeling, but the approach is entirely reliant on existing knowledge of enzyme kinetics and pathways and thus has limitations (Aurich and Thiele, 2016).

Stable isotope tracing. By labeling and following carbon through metabolic pathways, stable isotope tracing can address questions regarding the extent and pattern of flux through metabolic pathways (Buescher et al., 2015). Stable isotope tracers are non-radioactive and naturally occurring, and are therefore safe. The technique is typically destructive to the sample to enable mass spectrometry, but can be performed on cultured cells, tissues, and in vivo. NMR can instead be used for detection, with a significant sacrifice in sensitivity (Rodrigues et al., 2013). ^{13}C-containing molecules like glucose and glutamine have been used extensively, as the ^{13}C in the parent molecule is transferred to downstream metabolites that can be detected by mass spectrometry (Fig. 3D). The extent to which metabolites become enriched with ^{13}C combined with an analysis of the concentration of the metabolite is an indication of the flux through the metabolic pathway. The same, in principle, is true for ^{15}N- and ^{3}H-labeled substrates. For isotope tracing with cells in culture, a full complement of growth media is typically added with replacement of a fuel like glucose with ^{13}C-labeled glucose. The system is incubated for a sufficient time to allow the sample to come to isotopic steady state, and then the media is removed and the sample is extracted and derivatized for analysis by mass spectrometry. The spectral peaks for each metabolite are then used to quantify its abundance as well as the mass isotopomer distribution (the fraction of a metabolite enriched with zero, 1, 2, 3, etc ^{13}C-labeled carbons). From these data, as well as separate experiments using other metabolic tracers, the extent of

carbon flux from different substrates into the pool of a particular metabolite and its precursors can be calculated (Kelleher and Nickol, 2015). The ability to resolve cell-type specific metabolism in brain tissue or co-cultures is somewhat limited, and is reliant on either pharmacologic agents or genetic manipulation of isoforms of metabolite transporters or enzymes to control metabolism in particular cell types. Stable isotope tracing can determine the *relative* rates of flux of one labeled substrate versus another. Quantitative rates of flux can be determined by an extension of the technique termed *metabolic flux analysis* whereby the ^{13}C-labeling pattern of intracellular metabolites is pinned to the rates of uptake and release of metabolites from a biological system. The data is applied to a computational network model of biochemical reactions that enables quantitative estimates of flux through specific pathways (Buescher et al., 2015). The approach is more labor- and data-intensive than stable isotope tracing, and is reliant on a robust computational model that accounts for all potential reaction pathways.

Finally, in a particularly powerful approach, stable isotope tracing with mass spectrometry can be coupled with real-time measurements of metabolic flux using respirometry (a technique described in detail in Section C.1). The depth of insight provided by the combination of the two approaches is greater than either technique alone, and has been applied to the study of cultured cortical neurons (Divakaruni et al., 2017), the mouse retina (Du et al., 2016), and glioma metabolism and growth (Yang et al., 2014a).

C.8 Mathematical modeling

Mathematical modeling aims to integrate the outcome of multiple experimental techniques in order to grasp complex behaviors that are otherwise impenetrable. It serves to identify variables and parameters that are critical and should therefore be given experimental attention, to then guide the design of experiments by *a priori*, inexpensive visualization of possible outcomes. At its best, it may prefigure novel phenomena. In the specific case of brain energy metabolism, models combine structural parameters of brain tissue with the microscopic properties of enzymes and transporters in view of dynamic readouts obtained by technologies like PET, MRS, fMRI, tissue electrodes, microdialysis, and genetically-encoded nanosensors. A key well-substantiated assumption of current models is that metabolism is compartmentalized, that is, that it occurs in a few well-mixed reservoirs (say neuronal and glial). Thus, chemical and transport reactions can be represented by a small set of ordinary differential equations that are solved by numerical simulation using standard computers. The outcome of a model is as good as what the model is fed, and so different groups have arrived at opposite conclusions: witness the astrocyte-to-neuron lactate shuttle controversy (Hyder et al., 2006; Aubert and Costalat, 2007; Mangia et al., 2009; Jolivet et al., 2015). While debate speaks of a lively discipline, assumptions about key parameters are often hidden, and so it has been hard for the non-initiated to judge the relative merits of competing models (Jolivet et al., 2010). In view of structural, functional, and likely metabolic heterogeneity of neurons and glial cells (DeFelipe et al., 2013; Freeman, 2015), future models will have to become more complex, as has recently occurred for neuronal ATP subcellular domains (Le

Masson et al., 2014). The next generation of models will also have to deal with growing evidence for fast local regulation of enzymes, transporters and channels in response to neural activity (Barros et al., 2013). However, it is important to understand that a more complex model (i.e. which include more equations and more parameters) is not necessarily better to adequately describe the different possible outcomes of a system. It all depends on the question(s) that need(s) to be addressed.

BOX 1 The BOLD-fMRI method: relationship with energy metabolism

MRI is well known for its capacity to non-invasively provide high resolution images of brain structures. MRI can also be rendered sensitive to local magnetic field inhomogeneity. This principle has been applied to develop the most common modality of fMRI, where the so-called Blood Oxygenation Level Dependent (BOLD) effect on magnetic field inhomogeneity is detected during brain activity. The BOLD effect relies on the local transient increase of the oxyhemoglobin-to-deoxyhemoglobin ratio during the haemodynamic response associated with neuronal activity (Ogawa et al., 1990). Since deoxyhemoglobin induces magnetic inhomogeneity, activated regions of the brain, with less deoxyhemoglobin, exhibit a stronger BOLD signal. BOLD-fMRI is either used with task-based paradigms, in which the mean of the baseline state is subtracted from the mean of the stimulated state to identify activated regions associated with the task, or it is used to study the brain at rest, in the absence of an explicit task (resting state BOLD-fMRI; Gusnard et al., 2001). BOLD contrast fMRI has come to dominate the functional neuroimaging field.

The BOLD-fMRI signal is not a straightforward measure of energy metabolism. The deoxyhemoglobin content within a voxel is determined by the balance between the supply of oxygenated blood, the rate of oxygen consumption and the fractional blood volume. This is because the supply of oxygen is not matched precisely with the demand that the BOLD-fMRI signals change in regions where the actual amount of oxygen remaining in blood vessels is modified during activation. Hence, the BOLD response is dependent upon hemodynamic (blood flow and volume) and metabolic (oxygen consumption) responses. Although the value of BOLD fMRI for functional brain mapping is undisputed, the more subtle questions of how to interpret and use BOLD response magnitude and changes to derive information about energy metabolism are still unresolved. A reduction in the BOLD signal can result from a reduced neuronal activation, abnormal neuron/astrocyte coupling, a reduced vascular response, or a combination of all these components of the neurovascular response.

Quantitative fMRI techniques have been developed to measure the hemodynamic and metabolic responses to modulations in brain activity (Pike, 2012). They include arterial spin labeling (ASL) to measure variations in tissue perfusion in each pixel (which can help to disentangle confounding mechanisms), contrast agents to measure cerebral blood volume (CBV) and calibrated fMRI using biophysical models of the BOLD signal to estimate changes in the cerebral metabolic rate of oxygen consumption ($CMRO_2$). Calibrated fMRI may be used as a biomarker of brain disorders that could be applied for therapy monitoring.

Concluding Remarks

The diversity, range and depth of the techniques applied to the study of brain energy metabolism are amazing, as is the complexity of the problem in hand. As we are now gaining access to the particular metabolic behavior of specific brain areas, cell types and even individual cells, techniques are becoming more and more sophisticated, each own having its own domain, strengths and limitations (Table 1). A challenge for the near future is to increase the crosstalk between technical viewpoints, perhaps through the active exchange of ideas and collaboration between dedicated laboratories.

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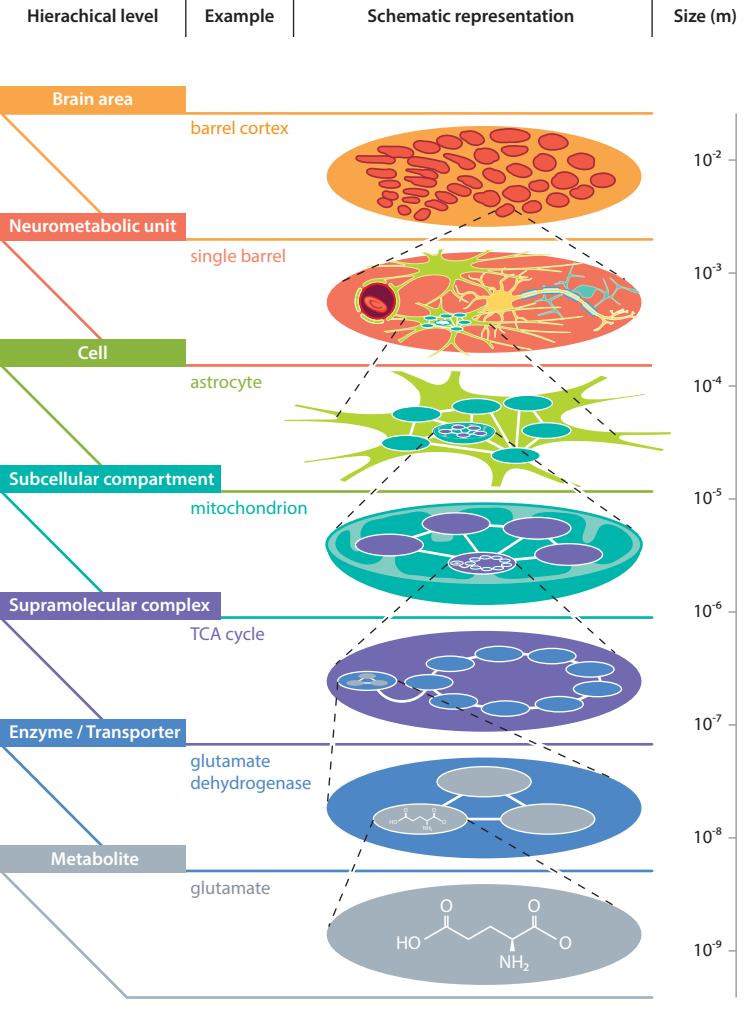
Figure legends

Figure 1. Hierarchical organization of brain energy metabolism. Structural levels result from binding forces becoming weaker as small structures accrete into larger ones but also have an evolutionary explanation (Simon, 1962). The hierarchical organization of brain metabolism explains the success of reductionist investigative approaches.

Figure 2 Technical domains in brain energy metabolism research. Different techniques perform over different domains, which are in turn defined by a combination of organizational level, experimental control and relevance to human physiology. For example, electrophysiology achieves excellent experimental control at small and intermediate hierarchies by *in vitro* recording from single channels, single cells or small cell assemblies, but it is not practical *in vivo* and cannot be used in humans. MRS, in contrast, may be applied to human subjects, but has limited experimental control and provides time- and space-averaged measurements.

Figure 3. Examples of recent data in brain energy metabolism. A. Astrocytes in organotypic hippocampal slice culture were transfected with cDNA constructs encoding for hexokinase-ECFP and GLT1-mCherry using a gene-gun. **B**. In vivo [^{14}C]-2-deoxyglucose uptake, obtained as described in Boussicault et al., 2014. **C**. ^{1}H-MRS data obtained in a rat, at rest or during whisker stimulation. The difference between the two conditions reveals an increase in tissue lactate. The BOLD fMRI response was elicited with the same paradigm. **D**. Rat neurons in culture were exposed to 3-$^{13}C_1$ glucose so that the labeled carbon is lost via the PDH reaction but retained by the anaplerotic reactions of pyruvate carboxylase (PC) and malic enzyme (ME), from Divakaruni et al., 2017. **E**. Perturbation of hippocampal fuel fluxes during a memory task (adapted from Newman et al., 2011). **F**. Lactate-mediated depolarization and firing of a neuron in the locus coeruleus after optogenetic stimulation of neighboring astrocytes (adapted from Tang et al., 2014). **G**. The graph show the biphasic response to astrocytic lactate levels as detected with Laconic during local stimulation of the somatosensory cortex (from Sotelo-Hitschfeld et al., 2015). The top image shows the expression of Laconic and Pyronic in neurons and astrocytes of the somatosensory cortex using viral vectors (adapted from Machler et al., 2016). Bottom images were taken from a transgenic mouse expressing ATeam in neurons described in Trevisiol et al., 2017.


Hierachical level
Example
Schematic representation
Size (m)
Brain area
barrel cortex
Neurometabolic unit
single barrel
Cell
astrocyte
Subcellular compartment
mitochondrion
Supramolecular complex
TCA cycle
Enzyme / Transporter
glutamate dehydrogenase
Metabolite
glutamate
HO
O
O
O
NH2
10-2
10-3
10-4
10-5
10-6
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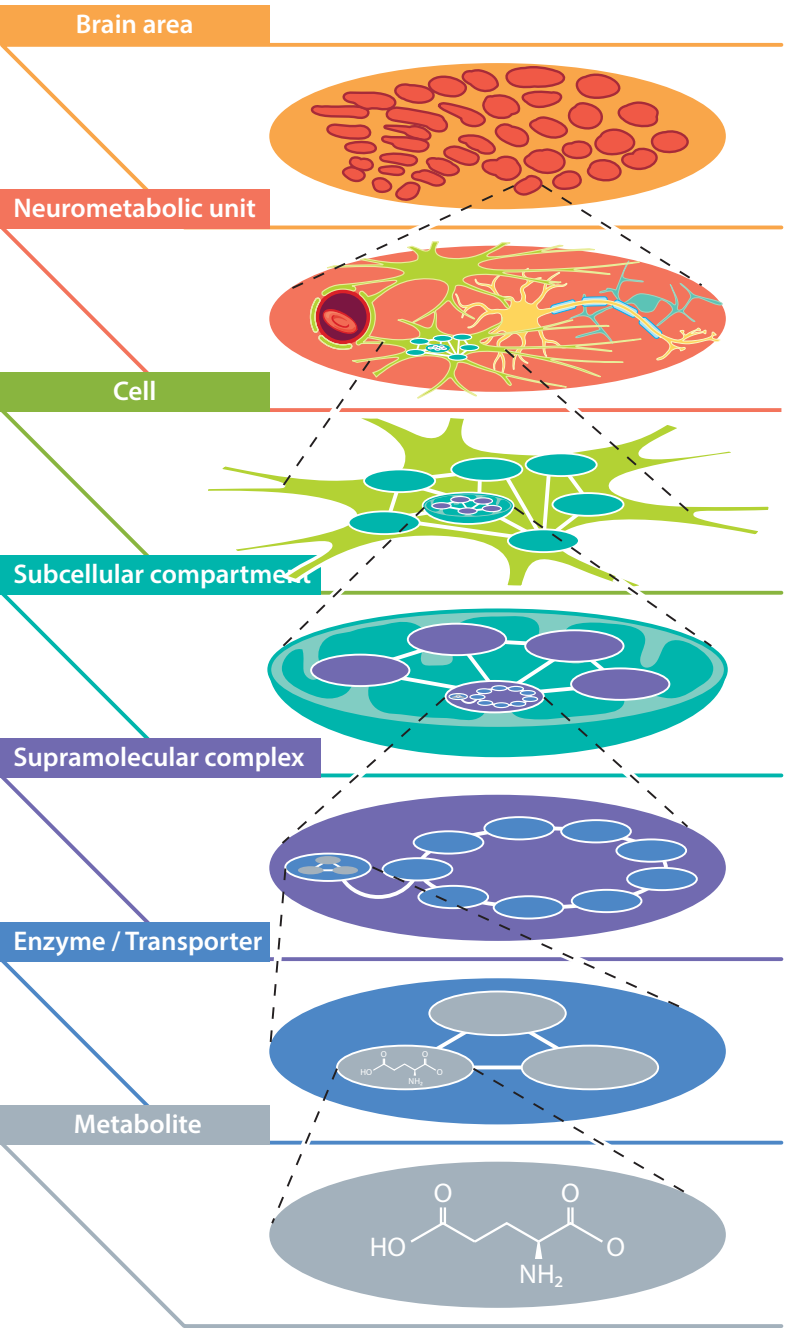
10-8
10-9



Non-human studies

Human studies

MRS

PET

fMRI

auto radiography

omics

micro sensors/dialysis

respirometry

fixed cell & tissue imaging

live cell & tissue imaging electrophysiology

protein-protein interaction detection

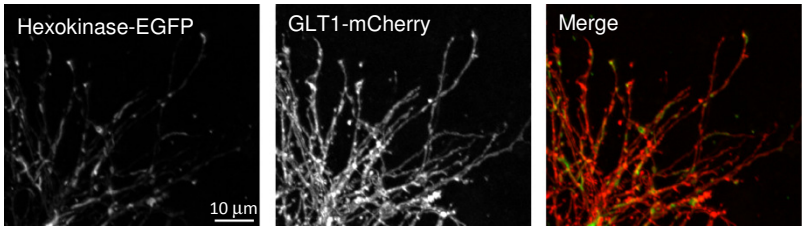
electron microscopy

X-ray difraction

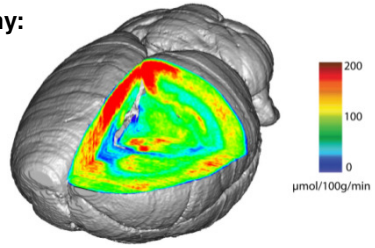
Experimental control

relevance for human physiology

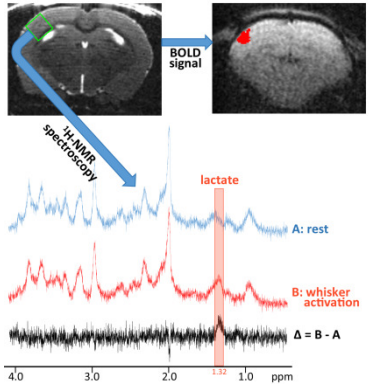
A **Confocal Microscopy:** Localization of enzymes and transporters in fine astrocyte processes.



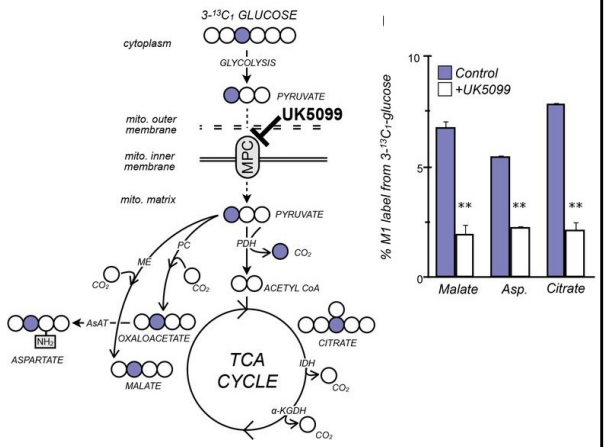
B **Quantitative 3D Autoradiography:** Local rate of glucose utilization throughout the rat brain.



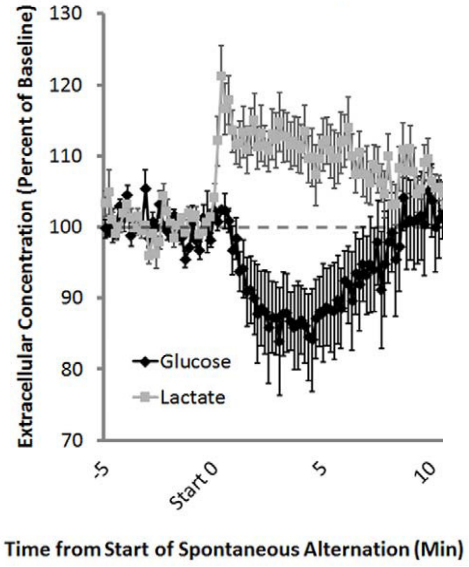
C **^{1}H-MRS:** Tissue lactate and BOLD response in the rat somatosensory cortex.



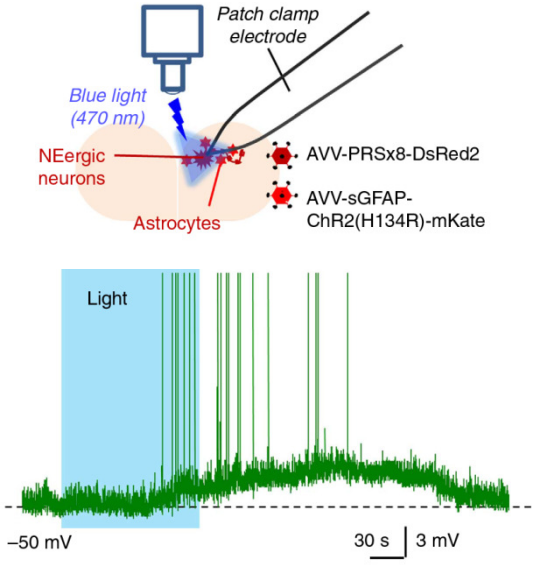
D **^{13}C-MRS:** Demonstration of glucose anaplerosis.



E **Enzyme-based microsensors:** Extracellular glucose and lactate dynamics in the rat hippocampus.



F **Optogenetic control:** Electrophysiological response of neurons to astrocytic stimulation.



G **Genetically-encoded nanosensors:** Astrocytic lactate dynamics in mouse somatosensory cortex and neuronal ATP sensor transgenic expresion.